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## ORIGINAL ARTICLE

# ALA- or Ce6-PDT induced phenotypic change and suppressed migration in surviving cancer cells

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**KEYWORDS**ALA-PDT;  
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migration ability;  
MMP9

**Abstract** *Background/purpose:* 5-Aminolevulinic acid mediated photodynamic therapy (ALA-PDT) has been used for the treatment of precancerous lesions and oral cancers. Compared with traditional treatment such as surgery, ALA-PDT can selectively kill cancer cells and reduce side effects. However, some cells might survive from PDT and may be directly attributable to the limited penetration of light. Therefore, it is necessary to elucidate alterations in cellular behavior and molecular mechanisms in cancer cells that have survived from PDT.

*Materials and methods:* Human tongue squamous carcinoma cells, SCC-4 cells, and mice bearing C26 tumors were used as cancer models *in vitro* and *in vivo*. After irradiation with the LD<sub>50</sub> (50% lethal dose) light dose, the ability of cellular migration and metastasis related gene expression were assayed.

*Results:* Cells were rounded up and migration ability was shown to have significantly decreased under the influence of irradiation with the LD<sub>50</sub> light dose. Gene expression related to metastasis including matrix metalloproteinases (MMPs) and chloride intracellular channel 4 (CLIC4) was also reduced. In addition, in the animal tumor model, mRNA expression of MMP9 and CLIC4 was also noticeably decreased.

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**Conclusion:** Our results indicate that PDT could reduce the expression of invasion related genes of surviving cancer cells *in vitro* as well as *in vivo*. These could be used as references for clinical PDT of oral cancer in the future.

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## Introduction

5-Aminolevulinic acid mediated photodynamic therapy (ALA-PDT) has been used to treat oral precancerous lesions and oral cancers including oral squamous cell carcinoma.<sup>1,2</sup> Compared with traditional treatment such as surgery, ALA-PDT is less invasive and can selectively kill cancer cells and reduce resultant side effects such as scar formation.<sup>3</sup> However, ALA-PDT may cause a sublethal effect on cancer cells due to the limited penetration of light. These survival cells may raise the risk of cellular metastasis.<sup>4</sup> Therefore, it is necessary to elucidate alterations in cellular behavior and molecular mechanisms in cancer cells survived from PDT.

PDT is a potential therapy for cancer treatment, which combines a nontoxic photosensitizer, oxygen and light, to generate cytotoxic species.<sup>5</sup> After light irradiation, the photosensitizer activates from a ground state to a higher energy excited state. The activated photosensitizer may subsequently react with molecules in its direct environment by an electron transfer process to form radicals, which can further interact with oxygen to produce reactive oxygen species, such as superoxide anion radical, hydrogen peroxide, and hydroxyl radical (type I reaction). The triplet photosensitizer may directly transfer energy to oxygen to also generate highly reactive singlet oxygen (type II reaction). Singlet oxygen is the major product of the photodynamic effect. Because of its short half-life and high activity, singlet oxygen can only diffuse and react with surrounding cells within 10–55 nm.<sup>6</sup> Besides, ALA itself is not a photosensitizer. While entering into a cell, it can be converted into the photosensitizer, Protoporphyrin IX (PpIX), in mitochondria resulting in damage to the cell after a 630-nm wavelength irradiation.<sup>7</sup> Compared with normal cells, cancer cells are more likely to uptake ALA and accumulate PpIX.<sup>7–9</sup> These cells utilize the photosensitizing agent that is able to photochemically eradicate malignant cells without hugely damaging circumjacent normal cells.

Previously, we have proven the satisfying efficacy of a sol-gel formulation of ALA in patients with oral verrucous hyperplasia lesions and extensive oral verrucous carcinoma. After treatment of the topical sol-gel form of ALA and irradiation with a 635-nm light-emitting diode (LED) light source, the lesion showed complete regression.<sup>10,11</sup> For the very common human papilloma virus which causes warts that can affect many different sites including the face, hands, feet, and genitalia, it was also demonstrated to be an effective and painless treatment.<sup>12</sup> Nevertheless, the penetration of light and the concentration of a photosensitizer still restrict the effect to the topical tissue. There is the risk that the deeper lesion converts to a more aggressive tumor via the ALA-PDT induced sublethal effect. The relationship among the epidermal growth factor receptor

(EGFR) and cell proliferation, angiogenesis, and metastasis has been proven.<sup>13,14</sup> The influence of ALA-PDT on the ability of invasion and EGFR expression patterns in surviving lung adenocarcinomas, melanoma, breast carcinoma, and head and neck cancer cell lines have been reported.<sup>15,16</sup> However, the cellular behavior and the molecular mechanism of metastasis related gene expression such as EGFR and matrix metalloproteinases (MMPs)<sup>17,18</sup> are not clear. It has been shown that reduced MMP9 and the change of invasive ability induced by PDT are related to down-regulation of chloride intracellular channel 4 (CLIC4).<sup>19,20</sup> In the current study, we evaluated the migration ability and metastasis involved genes such as EGFR, CLIC4, MMP2, and MMP9 in surviving cancer cells, which provide evidence that the alternation of cellular behavior resulted in decreased cellular metastasis in oral cancer cells survived from ALA-PDT. For application and to verify whether the cellular behavior of SCC4 cells is ALA-PDT or is oral cancer cell line specific, another type of photosensitizer, Chlorin e6 (Ce6), and C26 tumor-bearing mice were also used in this study. Ce6 is a type of photosensitizer compared with the first generation of photosensitizers, such as hematoporphyrin, which is more potent in tumor cytotoxicity, has stronger absorption at longer wavelengths, and has lower side effects in skin.<sup>21</sup> The purpose of this study is to verify the efficacy and metastasis related gene expression in PDT surviving cancer cells and the animal tumor model.

## Materials and methods

### Cell culture and photodynamic treatment

The human tongue squamous cell carcinoma, SCC-4, was cultured in DMEM/F12 (high-glucose Dulbecco's modified Eagle medium/nutrient mixture F-12; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) and 400 ng/mL hydrocortisone (Sigma-Aldrich, St Louis, MO). The murine colorectal cancer cell line, Colon-26 (C26), was grown in RPMI-1640 medium, supplemented with 10% FBS. The cultures were incubated at 37 °C in an atmosphere consisting of 5% CO<sub>2</sub> and 95% air.

For ALA-PDT, SCC-4 cells were cultured in dishes or plates and incubated with 1 mM ALA for 3 hours. Then cells were exposed to various doses of light. The light source consisted of high power LED array with the wavelength centered at 635 ± 5 nm.<sup>22</sup> The fluence rate of an LED light source is 60 mW/cm<sup>2</sup>.

For Ce6-PDT, after incubating C26 cells with 0.5 µg/mL Ce6 for 2 hours, cells were washed with phosphate buffered saline twice. Then, cells were irradiated by various doses of light with a diode laser (662 nm, 105 mW/cm<sup>2</sup>).

After 24 hours of incubation in the complete medium, cells were collected for further analysis.

### Experimental animals

Male BALB/cByJ mice (in the weight range of 17–20 g; 6–8 weeks of age) were purchased from the National Laboratory Animal Center (Taipei, Taiwan) and kept under controlled lighting (14 hours light/10 hours dark) at a constant temperature ( $23 \pm 2^\circ\text{C}$ ), with water and NIH 31 laboratory mouse food supplied *ad libitum*.

C26 cells were implanted in the right back of mice by subcutaneous injection ( $2 \times 10^5$  cells per mouse in  $50 \mu\text{L}$  of no phenol red RPMI-1640 medium). Mice were treated with Ce6 by intravenous injection through the tail vein when the tumor had grown to  $300 \text{ mm}^3$  in volume. The concentration of Ce6 was  $2.25 \text{ mg/kg}$ . Ce6 was diluted with  $0.9\% \text{ NaCl}$ . After administering the chemical and being kept in the dark for 6 hours, the mice were irradiated with a laser light (wavelength  $662 \text{ nm}$ , irradiance intensity  $95 \text{ mW/cm}^2$ ) and the energy density was  $100 \text{ J/cm}^2$ . After a period of 24 hours, the mice were sacrificed and tumors were collected. The tumor size was measured with an electronic balance and followed the determined gene expression in the tumors.

### 3(4,5-Dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolium bromide assay

After being irradiated with  $3 \text{ J/cm}^2$ ,  $6 \text{ J/cm}^2$ ,  $9 \text{ J/cm}^2$ , and  $12 \text{ J/cm}^2$  and then cultured in complete medium for 24 hours, cells were incubated with  $0.2 \text{ mg/mL}$  3(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution to examine cell viability. MTT was diluted with DMEM. After 2 hours, the formation of purple water insoluble formazan product was dissolved by dimethyl sulfoxide (DMSO) and then analyzed via a spectrophotometer at an absorbance of  $570 \text{ nm}$ . Cells exposed to serum-free, no phenol red medium but not light were used as control. MTT activity (%) = (mean absorbance of treated cells/mean absorbance of control cells)  $\times 100\%$ .

### Migration assay

To verify the cellular ability of migration by using transwell, cell suspensions ( $1 \times 10^5$  cells/well) were placed on the upper layer of a cell permeable membrane and a solution containing the complete medium was placed below the cell permeable membrane. Following incubation for 24 hours, the cells that migrated through the membrane were stained with crystal violet and counted.

### Gene expression

Total RNA was extracted from cells or tissue using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. cDNA was synthesized with random primers and SuperScript II reverse transcriptase (Invitrogen). The mRNA expression level of each gene was normalized to  $\beta$ -actin. The gene-specific primers were as follows: MMP9, 5'-GCCACCACCGCCAACTA-3' (forward) and 5'-GCCACGAGGAACAACTG-3' (reverse); MMP2, 5'-GCACCACTGAGGACTAGGA-3' (forward) and 5'-CATCTTCCGTCA

CTGCG-3' (reverse); EGFR, 5'-GATAGACGCAGATAGTCGC-3' (forward) and 5'-TTCTTCATCCATCAGGGCAC-3' (reverse); CLIC4, 5'-GCAGTGATGGTGAAAGCATAG-3' (forward) and 5'-TATAAATGGTGGGTGGGTCC-3' (reverse);  $\beta$ -actin 5'-TGGACTTCGAGCAAGAGATGG-3' (forward) and 5'-ATCTCC TTCTGCA-TCCTGTCG-3' (reverse).

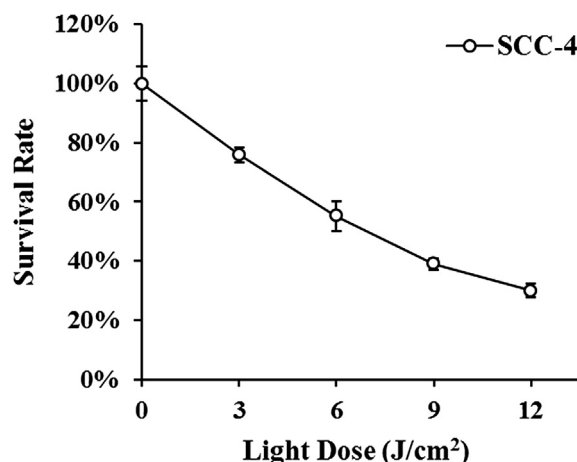
### Statistical analysis

Statistical analysis was conducted using a one-way analysis of variance with Dunnett's *post hoc* test using GraphPad InStat software, version 3.00 for Windows (GraphPad Software, San Diego, CA, USA).

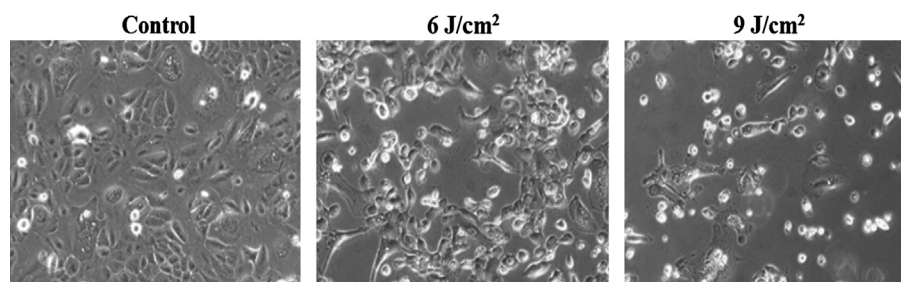
## Results

### Sublethal dose of 5-aminolevulinic acid mediated photodynamic therapy

To investigate the sublethal effect on SCC-4 cells, the sublethal dose of ALA-PDT was verified first. After being seeded in a 96-well plate overnight and further incubated with  $1 \text{ mM}$  ALA for 3 hours, SCC-4 cells were irradiated by  $3 \text{ J/cm}^2$ ,  $6 \text{ J/cm}^2$ ,  $9 \text{ J/cm}^2$ , and  $12 \text{ J/cm}^2$  with the LED array ( $635 \pm 5 \text{ nm}$ ,  $60 \text{ mW/cm}^2$ ). Then, the MTT assay was used to examine the survival rate of SCC-4 cells. As shown in Fig. 1, the decrease in survival rate on SCC-4 cells after ALA-PDT treatment related to the increasing of light dose (light dose-dependent manner) and the  $\text{LD}_{50}$  (50% lethal dose) was kept at around  $6 \text{ J/cm}^2$ . We also observed the morphology of these cells via a phase contrast microscope (Fig. 2). With the increase of the light dose, the cell number was diminished and cells were rounded up significantly



**Figure 1** The survival rate of SCC-4 cells after ALA-PDT with various doses of light. Cells were incubated with  $1 \text{ mM}$  ALA and then exposed to light irradiation at different light fluence. Cell viability was assessed by MTT assay 24 hours after light irradiation. Data are mean  $\pm$  standard error of the mean obtained from three independent experiments. ALA-PDT = 5-Aminolevulinic acid mediated photodynamic therapy; MTT = 3(4,5-dimethyl-thiazoyl-2-yl)-2,5-diphenyltetrazolium bromide; SCC = squamous carcinoma cells.

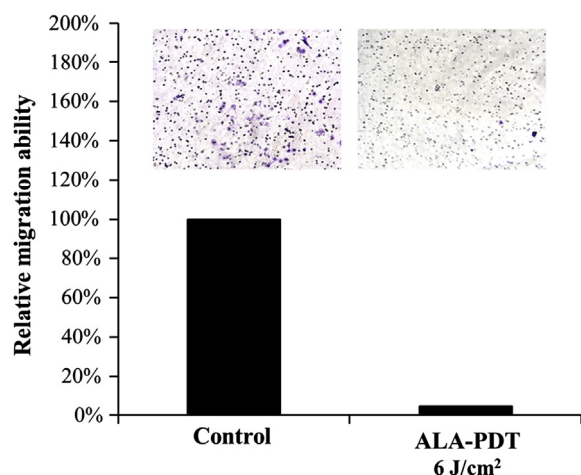


**Figure 2** The morphology of SCC-4 cells after ALA-PDT. Cells were observed by using phase contrast microscopy. Under the light dose of 6 J/cm<sup>2</sup> and 9 J/cm<sup>2</sup>, cells were rounded up and the number of cells was significantly decreased. ALA-PDT = 5-Aminolevulinic acid mediated photodynamic therapy; SCC = squamous carcinoma cells.

revealing the possibility that there is a change in the cellular ability of migration.

### Migration ability

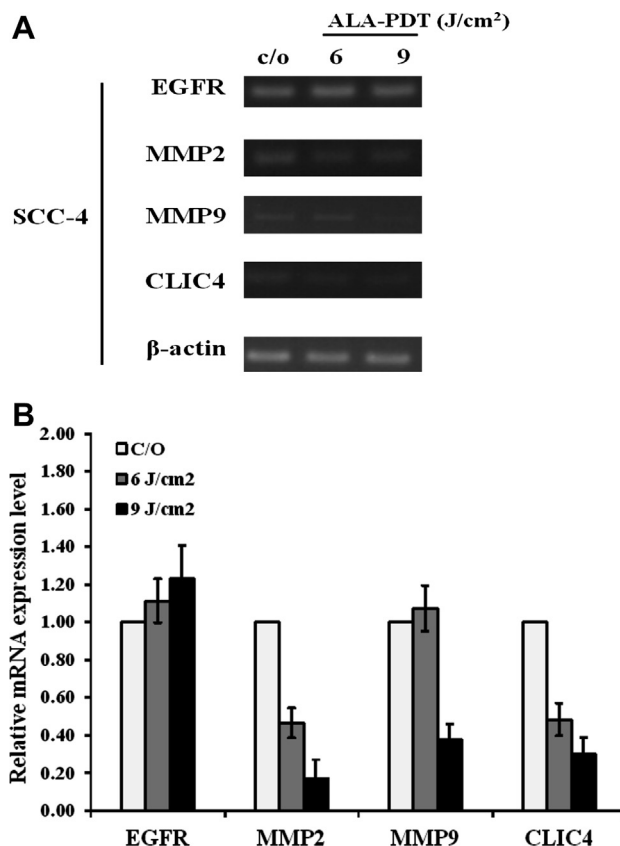
The ability of migration was assayed by using the transwell migration assay. After treating SCC-4 cells with ALA-PDT under the light dose of 6 J/cm<sup>2</sup>, the surviving cells were placed on the upper layer of the transwell. Twenty-four hours post-incubation, the migration rate was measured via being stained with crystal violet and then counting the migrated cells. The number migrated was normalized to control SCC-4 cells. As shown in Fig. 3, as compared with control cells, the relative cellular migration ability was decreased to 4.4%.



**Figure 3** Cellular migration ability under the influence of ALA-PDT. Representative images (top) of transwell migration assay using complete medium (10% FBS) as chemoattractant showed the migration ability of control and ALA-PDT (6 J/cm<sup>2</sup>) treated SCC-4 cells. The migrated cells were stained with crystal violet and counted. Results were obtained from three independent experiments. The relative fold change in the number of migrated cells is shown (below), with the results from control cells given as 100%. ALA-PDT = 5-Aminolevulinic acid mediated photodynamic therapy; FBS = fetal bovine serum; SCC = squamous carcinoma cells.

### Metastasis related gene expression

As ALA-PDT could reduce the migration ability and change the shape of SCC-4 cells, we further verified the expression level of metastasis related genes. After the excretion of total RNA from LD<sub>50</sub> (6 J/cm<sup>2</sup>) and LD<sub>75</sub> (9 J/cm<sup>2</sup>) of ALA-



**Figure 4** The expression levels of MMP2, MMP9, and CLIC4 were reduced in cells treated with PDT. mRNA expression of MMP2, MMP9, and CLIC4 in SCC-4 cells 24 hours post-ALA-PDT under different light dose was assayed. (A) Reverse transcription polymerase chain reaction (RT-PCR) results; (B) densitometric analysis. Data represent three independent experiments, mean  $\pm$  standard deviation. ALA-PDT = 5-Aminolevulinic acid mediated photodynamic therapy; CLIC4 = chloride intracellular channel 4; MMP = matrix metalloproteinase; SCC = squamous carcinoma cells.



PDT treated surviving cells, specific primers of EGFR, MMP2, MMP9, and CLIC4 were used for polymerase chain reaction (PCR). The mRNA expression level was calculated and then normalized to  $\beta$ -actin for lessening the initial variation in sample concentrations (Fig. 4). Cells only exposed to ALA without light were used as the control (ALA only). The mRNA expression level of MMP2, MMP9, and CLIC4 was shown in a light dose-dependent manner that decreased depending on the increasing light dose. These results demonstrated that cancer cells survived from ALA-PDT exhibit reduced migration and were also related to down-regulation of MMP2, MMP9, and CLIC4. However, there was no significant difference between the control and ALA-PDT treated surviving cells.

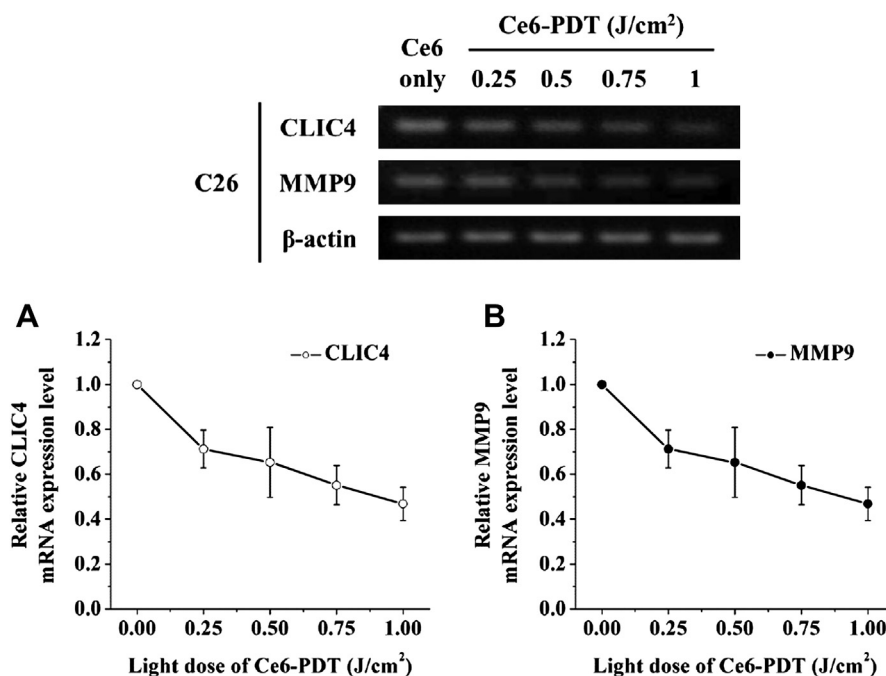
### Ce6 mediated photodynamic therapy

To investigate whether the change of metastasis related gene expression is ALA-PDT specific and for clinical application, Ce6 was used as a photosensitizer for photodynamic treatment. After being incubated with Ce6 for 2 hours, C26 cells were irradiated by 0.25 J/cm<sup>2</sup>, 0.5 J/cm<sup>2</sup>, 0.75 J/cm<sup>2</sup>, and 1 J/cm<sup>2</sup>. Twenty-four hours later, the total RNA of survival cells was extracted for analysis of the expression pattern for MMP9 and CLIC4. Cells were exposed to Ce6 but no irradiation was used as the control (ALA only). The decrease of CLIC4 expression was also highly related to increasing light doses and MMP9 (Fig. 5). These results are consistent with ALA-PDT survived SCC-4 cells. Furthermore, we used BALB/cByJ mice bearing C26 tumors as the animal model for demonstrating metastasis associated gene

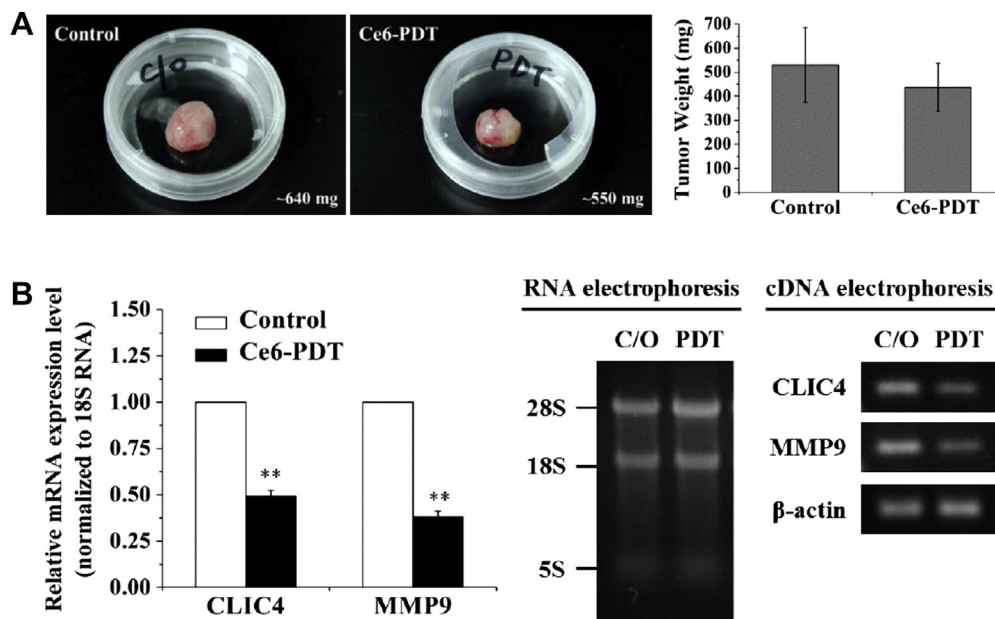
expression *in vivo*. Mice were treated with Ce6 at a dose of 2.25 mg/kg Ce6 by intravenous injection through the tail vein when the tumor was grown to 300 mm<sup>3</sup> in volume. Mice injected with the same volume of 0.9% NaCl was used in the control group. Six hours later, mice were irradiated with laser light (wavelength 662 nm, irradiance intensity 95 mW/cm<sup>2</sup>) and the energy density was set at 100 J/cm<sup>2</sup>. Twenty-four hours post-irradiation, the mice were sacrificed and the tumors were collected for determining the tumor size and gene expression level. As shown in Fig. 6A, as compared with the control mice, the tumor size of the Ce6-PDT treated mice decreased by approximately 15%. CLIC4 and MMP9 mRNA expression was significantly reduced ( $P \leq 0.01$ ; Fig. 6B). These results indicate that PDT could reduce the expression of invasion related genes of surviving cancer cells *in vitro* as well as *in vivo*.

### Discussion

Under the influence of PDT, cellular migration and attachment were suppressed in many cell lines.<sup>23,24</sup> It has also been proven that PDT results in a decrease in cellular invasion in lung adenocarcinomas, melanoma, breast carcinoma, and head and neck cancer cells lines.<sup>15,16</sup> Besides, the metastasis of highly related molecular cells, MMP2 and MMP9, which show the ability to degrade the basement membrane and extracellular matrix to promote the process of migration and invasion,<sup>17,18</sup> could be affected by PDT.<sup>25–27</sup> However, the mechanism with which PDT regulates the alteration of MMP2 and MMP9 expression is still unclear. Recently, we have shown that downregulation of



**Figure 5** The expression levels of MMP9 and CLIC4 were reduced in C26 cells treated with Ce6-PDT: after incubation with 0.5  $\mu$ g/mL Ce6 and exposure to light irradiation at different light fluence. MMP9 and CLIC4 expression levels were analyzed via reverse transcription polymerase chain reaction. (A,B) Densitometric analysis. Statistical analysis was performed according to the data from three independent experiments. The results represent the mean  $\pm$  standard error of the mean for each group and control cells are given as 1.0. Ce6 = chlorin e6; CLIC4 = chloride intracellular channel 4; MMP = matrix metalloproteinase; PDT = photodynamic therapy.



**Figure 6** Ce6-PDT *in vivo*. Ce6 was administrated through tail vein injection into BALB/cByJ mice bearing the C26 tumor. Six hours later, 100 J/cm<sup>2</sup> of light was applied. After 24 hours, mice were sacrificed and tumors were then collected. Mice injected with the same volume of 0.9% NaCl were used as the control group. (A) Tumor size and (B) mRNA expression of MMP9 and CLIC4 ( $n = 3$ ). These results are represented as the mean  $\pm$  standard deviation for each group. \*\* $P < 0.01$ . Ce6 = chlorin e6; CLIC4 = chloride intracellular channel 4; MMP = matrix metalloproteinase; PDT = photodynamic therapy.

CLIC4 is related to reduced MMP9 and suppressed invasiveness induced by PDT in human lung adenocarcinoma, melanoma cells, etc.<sup>19</sup> In addition, CLIC4 has the ability to regulate the proliferation and differentiation as well as to assist the angiogenesis in tumor cells.<sup>28,29</sup> Tumor–stroma crosstalk signaling, which promoted the conversion of fibroblast to a more aggressive stromal myofibroblast, and the relationship with tumorigenesis of CLIC4 were also reported.<sup>20,30,31</sup> In this study, the suppressed migration and CLIC4, MMP2, and MMP9 expression proven in ALA-PDT caused sublethal SCC-4 cells. Furthermore, the reduction of gene expression was shown in a light dose-dependent manner. It suggests that the suppression of cellular migration may depend on the efficacy of ALA-PDT.

After ALA-PDT treatment, SCC-4 cells rounded up noticeably (Fig. 2; 9 J/cm<sup>2</sup>). It seems similar to the disorganization of the cytoskeleton in cells resistant to PDT.<sup>32</sup> The rearrangement of the cytoskeleton was modulated by the FAK (focal adhesion kinase)–Src kinase complex and associated with the alternation of cell motility and focal contact.<sup>33</sup> Inhibition of the phosphorylation of FAK, Src kinase, and its downstream extracellular signal-regulated kinase by sublethal PDT in head and neck cancer cell lines has been reported.<sup>16</sup> The rounded morphology induced by PDT also seems involved in migration suppression in SCC-4 cells. The alternation of the cytoskeletal arrangement and the molecular mechanism in ALA-PDT induced sublethal SCC-4 cells needs more investigation.

In PDT-treated cells, downregulation of EGFR has been notable *in vitro* and *in vivo*.<sup>15,34</sup> Overexpression of EGFR is common in many types of human tumors and is often correlated with the enhancement of cellular proliferation and metastasis.<sup>35,36</sup> Compared with parental SCC-4 cells, the surviving cells showed significantly suppressed cellular

migration (Fig. 3). Surprisingly, the expression of EGFR mRNA was not decreased like CLIC4, MMP2, and MMP9 (Fig. 4). Both EGFR-dependent and EGFR-independent mechanisms of promoting the growth and survival were found in clinical specimens from head and neck squamous cell carcinoma patients and oral squamous cell carcinoma animal models.<sup>37,38</sup> These may be associated with the rendering of the EGFR inhibitor not applicable as a single therapeutic agent<sup>39</sup> and the uniformity of the EGFR expression pattern in our results.

The results of Ce6-PDT showed that reduced CLIC4 and MMP9 expression is not ALA-PDT specific and the possibility of a suppressed migration of cancer cells survived from Ce6-PDT *in vivo*. PDT seems to have the potential of inhibiting the invasiveness and metastasis of cancer cells, which means it could have great potential for the treatment of oral precancerous lesions and oral cancers.

## Conflicts of interest

The authors have no conflicts of interest relevant to this article.

## Acknowledgments

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